Examining Pathological Effects of Hepatic Inflammation after Traumatic Spinal Cord Injury

Research Thesis

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Abstract

Sensory and motor functions of the body are regulated by the spinal cord, and traumatic spinal cord injury (SCI) permanently impairs these basic functions. The long term consequences of SCI include chronic medical conditions altering each patient's health and quality of life. In addition to the loss of proper sensory and motor function, injury to the spinal cord disrupts homeostasis of directly innervated peripheral organs such as the liver. Previously our lab showed that chronic pathological changes in the liver following SCI include enhanced lipid deposition and activation of resident liver macrophages called Kupffer cells (KCs) leading to chronic hepatic inflammation. Interestingly, after SCI the hepatic inflammatory response precedes intraspinal inflammation, and some reports suggest hepatic inflammation leads to exacerbated spinal cord lesion pathology. However, the ability of hepatic inflammation to alter chronic SCI outcomes has never been tested. Therefore, the current study examines the effects of exacerbated or attenuated liver inflammation after SCI. Liver inflammation was induced using a total bile duct ligation (BDL) model in a "gain of function" experiment to upregulate KC-mediated inflammation in the liver prior and subsequent to a mid-thoracic SCI. Liver tissue showed an upregulation of cytokine mRNA expression, KC activation, fibrosis, and lipid deposition at 28 days post-SCI when BDL preceded SCI compared to naïve and BDL Sham-operated animals. This reveals that injury to the spinal cord exacerbated the effects of BDL on liver pathology. However, no significant changes in spinal cord lesion size or immune activation were observed between the groups. A "loss of function" experiment showed depletion of KCs using gadolinium chloride ($GdCl_3$) occurs within 48 hours after injection. This model will be used to test if KC depletion improves outcomes after SCI. These studies support the link between hepatic and CNS inflammation and open the door for new therapeutic targets to improve quality of patient health and recovery in a clinical SCI setting.

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Introduction

A lifelong condition affecting the lives of thousands across the United States annually, spinal cord injury (SCI) is a traumatic neurological impairment that significantly reduces health and quality of life for patients. An estimated 10 to 40 individuals per million in the developed world are affected by SCI with 276,000 patients in the US alone[1–3]. Immediately following SCI the spinal cord tissue which survives the injury becomes largely at risk of secondary damage caused by neuroinflammatory mechanisms[4]. The spinal cord innervates the peripheral organs of the body, and secondary damage to the cord leads to chronic dysfunction due in part to the vast repertoire of infiltrating leukocytes across the damaged blood-spinal cord barrier[4–7]. The destruction of spinal tissue can lead to chronic health impairments aside from paralysis, including bladder infections, bowel dysregulation, and irregular respiratory and cardiac function[2]. Although the initial impact that displaces and injures the spinal cord tissue is substantial, there is also an intense inflammatory response ravaging the body. Inflammation after SCI upregulates the release of pro-inflammatory molecules and leukocytes into peripheral tissues, the circulation, and spinal cord itself[4,5]. Previous studies from our laboratory indicated that the liver, which is a critical immune and metabolic organ, is negatively affected by SCI leading to elevated lipid deposition and activation of Kupffer Cells (KCs), the resident macrophages of the liver[5,8]. The liver is a principle, visceral immune organ involved in the inflammatory response after central nervous system (CNS) injury, and drives the acute phase response (APR) that follows immediately[9–11]. That being said, relatively few studies examine the interactions between the liver and spinal cord after CNS injury—in particular the potential concomitant role of hepatic inflammation exacerbating SCI lesion pathology and peripheral deficits. Therefore, the goal of the current study is to assess the effects of induced hepatic inflammation on outcomes following traumatic SCI by way of "gain of function" and "loss of function" studies.

Hepatic and CNS inflammation are two distinct conditions increasingly studied in conjunction. As previously discussed, recent findings corroborate the CNS inflammatory response is potentiated by the liver's upregulation of the hepatic APR after SCI. The APR is an early reaction in an organism to a stimulus such as infection, necrosis, or trauma[9]. The hepatic APR induces an array of changes in the body including upregulation of leukocytes, fever, and changes in levels of pro-inflammatory cytokines, chemokines, and glucocorticoids. Though these inflammatory mediators facilitate damage caused by the APR, the theoretical purpose of this reaction is to promote a return to homeostasis by promoting tissue repair. Conversely, in the context of SCI, upregulation of hepatic cytokines like tumor necrosis factor (TNFα) and interleukin-1 beta (IL-1β) leads to enhanced leukocytosis in the bone marrow and the distribution of newly generated immune cells rapidly (~30 minutes) into the liver parenchyma as well as other peripheral organs[6]. This response most likely contributes to the development of multiorgan

dysfunction syndrome (MODS), systemic inflammatory response syndrome (SIRS), and inevitably metabolic syndrome in chronic SCI patients[5,6,12]. This is relevant to clinical SCI patients as metabolic syndrome affects organs such as the liver and their lifestyle as a result. Indeed, the APR of the liver not only affects the spinal cord following trauma, but also contributes to the exacerbation of peripheral organ dysregulation.

Kupffer cells in the liver contribute to the intensity of the hepatic APR preceding CNS injuries[8,13]. These resident mononuclear cells play a role in the phagocytosis of debris and, more importantly, serve as a major site of pro-inflammatory cytokine synthesis[14] and aid in the recruitment of peripheral monocytes to the parenchyma[15]. The downstream effects of enhanced hepatic immune cell function and inflammation were explored supplementary to brain injury[10,11], and the results showed an exacerbated CNS pathology due to enhanced synthesis of hepatic chemokines and consequently leukocyte recruitment. Some reports suggest that the lesion pathology of the injured spinal cord may be exacerbated by initial liver inflammation preceding intraspinal inflammation[5,16]. To effectively understand the relationship between lesion pathology and the immune response of the liver, it is essential to study both of these reactions jointly. Investigating the effects of heightened inflammation of the liver as well as the attenuation of the APR post-SCI can provide insight on the immune mechanisms leading to chronic injury sequelae. Establishing a better understanding of the secondary injury response will provide vital information for future work and possibly lead to novel therapeutic strategies to reduce lesioned tissue and improve quality of life by attenuating peripheral injuries and complications.

Background

Trauma to the spinal cord exhibits distinct pathological responses following the primary injury. As discussed before, the milieu of the injured spinal cord is significantly different in appearance when juxtaposed to the CNS of a healthy individual. The two branches of the nervous system—central and peripheral—communicate as peripheral nerves extending from the spinal cord directly innervate viscera and the muscles of the body. This transmission of information is disrupted by traumatic events causing SCI and after effects including bleeding, edema, activation of neuroglia, cytokine synthesis, and destruction of the blood-spinal cord barrier (BSCB)[2,17,18]. The lesioned spinal tissue resulting from the mechanical trauma is dysfunctional and can no longer maintain homeostasis with the innervated periphery. Loss of the blood-spinal cord barrier occurs in part by extravasation of leukocytes. These blood-borne immune cells along with neuroglia attempt to prevent further histological damage by sealing off lacerated tissue to form the glial scar around the lesion epicenter[19]. The regrowth of lesioned axons is hindered by this scarring while demyelination and axonal injury from activated resident microglia and recruited macrophages further worsen post-traumatic secondary damage[17]. Long term

absence of proper autonomic control from the spinal cord is detrimental to visceral organs[20], in particular to the liver. CNS control of the liver is mediated by sympathetic splanchnic nerves originating from level T7-T12 in the intermediolateral cell column of the spinal cord and from descending parasympathetic pathways in the brainstem. Spinal cord injury disrupts the normal propagation of signals from these autonomic pathways and can inhibit appropriate liver activity[21,22]. The dysfunction of the liver can be augmented further by the heightened immune response after injury. The purpose of the hepatic APR following SCI is to regain a homeostatic balance by attenuating the dysregulation of the liver, yet its upregulation could contribute to the limited repair and exacerbate pathological changes in the SCI lesion. While enabling SCI patients to walk again is the main objective in many spinal cord injury research projects, the sequelae that persist chronically such as autonomic dysfunction and complications or failure of metabolic, cardiovascular, and renal systems are given little attention yet contribute to patient mortality at a much higher rate[1,20]. These impairments are certainly not the primary focus of most studies, but are important inquiries that receive little attention in the SCI paradigm.

Previous findings from our laboratory have established that injury to the spinal cord induces pathological changes in the liver including enhanced lipid deposition, droplet size, chronic macrophage activation, and pro-inflammatory cytokine mRNA expression. Patients with spinal cord injuries are 43% more likely to show signs of nonalcoholic fatty liver disease (NAFLD) when compared to healthy individuals[5]. NAFLD essentially is observed as the hepatic manifestation of metabolic syndrome in chronic SCI patients[23]. Changes in lipid expression and inflammation in hepatic tissue after SCI can also lead to symptoms that are consistent with the advanced form of NAFLD known as nonalcoholic steatohepatitis (NASH) and proceeds to enhanced fibrosis, cirrhosis, and loss of regenerative capabilities in hepatic tissue[5]. A culprit of the aforementioned conditions is activated Kupffer cells that secrete cytokines like TNFα and concordantly hinder hepatocyte lipid metabolism[24]. These changes in the liver after SCI are detrimental, and current data show metabolic problems are contributing to mortality an increasing rate in clinical cases[1].

The liver and CNS are immunologically linked in the context of injury, and experimental hepatic inflammation activates microglia even in the non-injured CNS[12]. Subsequent to SCI, hepatocytes and Kupffer cells promote the acute inflammatory response by secreting cytokines and acute phase proteins into the bloodstream[6,8]. As cytokines are released into the circulation, the upregulation of leukocyte production in primary lymphoid tissue such as the bone marrow may drive increased immune cell recruitment to the spinal cord lesion epicenter. One group determined that levels of hepatic chemokines CCL-2 and CXCL-1 were observed in the blood as early as 2h and peaked at 4h after spinal cord compression injury[11]. The enhancement of the hepatic APR following injury in the spinal cord therefore could potentially exacerbate spinal inflammation which can feedback and produce further inflammation in the liver. In metabolic syndrome manifesting post-SCI, the liver perpetuates the deposition of lipids and cytokine synthesis while simultaneously

being damaged by inflammation resulting from these molecular events[5]. Therefore, spinal cord injury induces pathological changes in the liver and significantly disrupts proper metabolic function. Still in question however is what effect does changing hepatic inflammation, either through a "gain of function" experiment by induction of inflammation or a "loss of function" experiment attenuating inflammation, cause in SCI lesion pathology, peripheral immune activation, and functional recovery.

Preliminary data collected by our group observed the effects of induced hepatic inflammation on outcomes following spinal cord injury. We chose to employ the bile duct ligation (BDL) model to efficiently produce sustained, chronic hepatic inflammation caused by prolonged cholestasis. Cholestasis-induced inflammation enhances Kupffer cell activation, which induces acute phase protein production and cytokine synthesis in the inflamed liver, is observed in injuries of multiple etiologies[12,14,25,26]. Accumulation of bile acids in the biliary tract leads to irreversible fibrosis from proliferating stellate cells and substantial inflammatory damage to the liver tissue[27]. Hepatocytes and Kupffer cells exacerbate this damage by triggering the production of inflammatory mediators such as cytokines, chemokines, and adhesion molecules[15]. Our work showed enhanced pro-inflammatory gene expression was evident after 5d following BDL in rats. Hepatic macrophage expression and changes in lipid deposition around vasculature of the inflamed liver occurs after bile duct ligation and SCI. Interestingly in the spinal cord, spared tissue varied among experimental groups with the BDL and SCI group having the least amount of spared tissue and the most pronounced macrophage activation in the lesion epicenter (data not shown). Indeed, induced hepatic inflammation could exacerbate intraspinal inflammation and the NAFLD/NASH-like presentation in the liver after SCI as previously described[5]. This experimental "gain of function" in the liver can potentially affect lesioned spinal tissue in a feed-forward manner as well as incur further pathology to hepatic tissue.

Conversely, "loss of function" experiments that attenuate liver inflammation prior to SCI will be performed with the aim of dampening chronic peripheral impairments and neuroinflammatory responses. Gadolinium chloride ($GdCl_3$) is a compound widely used to deplete the liver Kupffer cells and inhibit phagocytosis and cytokine production thereby reducing exaggerated, KC-mediated inflammation[14,28,29]. Attenuation of the KC response in the initial hours following SCI may protect the spinal cord via reduced acute phase inflammation from the liver. Indeed, Kupffer cell activity contributes to the number of leukocytes recruited to the injured spinal cord and essentially facilitates secondary damage occurring immediately afterward[10,11]. The use of $GdCl_3$ also stimulates a reduction in the fibrosis indicative of liver injury and enhances hepatic tissue regeneration by inhibiting these macrophages[29,30]. Prevention of Kupffer cell activation also hinders steatosis of the liver by sparing hepatocytes, promoting lipid metabolism[31] and reversing NAFLD/NASH[32]. This could potentially prevent pathological changes in the liver in chronic SCI. It is essential to study the opposite effects of liver inflammation as a preventative measure to

reduce the sequelae of SCI such as NASH and possibly preclude inflammation and secondary damage to the spinal cord itself.

The current investigation will expand the examination of negative effects of induced inflammation with both histological and molecular analyses in the liver during the chronic phase of injury. These "gain of function" studies will explore changes in liver lipid deposition and spared tissue seen following spinal cord injury as well as hepatic and CNS macrophage expression. In addition, we will determine if induced hepatic inflammation prior to mid-thoracic SCI worsens outcomes. The outcome after selective depletion of Kupffer cells in SCI using $GdCl_3$ injections in a "loss of function" study will determine if the immune response of the liver is attenuated and thereby lessens intraspinal pathology. The link between hepatic and spinal cord inflammation after injury potentially has clinical relevance if attenuation of the hepatic APR is neuroprotective post-SCI. Collectively, these studies will contribute to and improve our understanding of liver inflammation in the context of spinal cord injury and its peripheral consequences in the chronic injury phase.

Methods

Bile Duct Ligation

All procedures were approved for use by the IACUC and followed set standards established by the NIH and The Ohio State University animal care guidelines. Total bile duct ligation (BDL) surgery was performed on female Sprague-Dawley rats to model biliary acid accumulation and presentation of hepatic inflammation due to chronic cholestasis. Rats (200-300 g; Harlan, Houston, TX) were anesthetized by an intraperitoneal injection of a solution of ketamine (80 mg/kg) and xylazine (10 mg/kg) and underwent a single ligation of the common bile duct or sham surgery. The abdominal cavity was opened, and the bile duct was exposed and ligated with a single suture superior to the duodenum. Sham surgeries exposed the bile duct only. The abdominal cavity was then stapled closed and the animals were allowed to recover in warm cages. The animal weights were monitored daily and any rats that lost more than 15% of their body weight were sacrificed. Typically the rats exhibited jaundiced skin and discolored urine and feces as early as 3 days post-BDL until the end of the experiment.

Spinal Cord Injury Methodology

Spinal cord contusion was performed on all animals as a clinically relevant model for SCI. At 5 days post-BDL, animals were anesthetized using ketamine and xylazine in the same way as the bile duct surgery. A dorsal laminectomy was performed at level 8 of thoracic vertebrae (T8). Spinal contusion injuries were administered using the Infinite Horizons device (Precision Systems and Instrumentation) at 200 kD of force. Back muscles were sutured and the skin was closed with wound clips. Rats recovered in warm cages after

closing the injury site and received 5 mL of saline solution by subcutaneous administration. Rat bladders were expressed twice daily and the animals were hydrated with daily subcutaneous 0.9% saline for the duration of the experiment after SCI.

Tissue Extraction and Preparation

Rats were sacrificed at 28 days post-BDL/23 days post-SCI. Fresh liver samples were collected from the right medial lobe and cut into 0.5g pieces that were flash frozen in liquid nitrogen. The rats were then perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer solution (PBS). The tissue was fixed for analysis as the PFA stabilized the samples by cross-linked polymerization. Liver and spinal cord tissue was removed and post-fixed in 4% PFA for 2 hours and transferred to 0.2 M phosphate buffer (PB) overnight. Samples then were removed from PB and cryoprotected for 2-3 days in 30% sucrose solution. Fixed liver and spinal cord samples were then frozen on dry ice and blocked using optimal cutting temperature (OCT) solution. Both the spinal cord and liver tissue were sliced in a cryostat and mounted on slides (Superfrost Plus Slides, Fisher Scientific) at 10 and 20 μm, respectively. The tissue was cut at -20°C and slides were stored at this temperature prior to immunohistochemistry (IHC) analysis.

Gadolinium Chloride Administration

Kupffer Cells numbers were reduced in a separate cohort of female, 250g Sprague Dawley rats in order to decrease inflammation of the liver. Animals were anesthetized using isoflurane (5%) and 1 mL of gadolinium chloride (10 mg/kg) dissolved in 0.9% saline or vehicle was injected and via the lateral tail vein. Rats were sacrificed and perfused 48 hours post-injection and tissue samples were collected as previously described.

Liver and Spinal Cord Histological Analysis

IHC was performed on liver and spinal cord tissue to assess local and peripheral effects of Kupffer Cell-mediated inflammation. OX42 (CD11b) antibody (Serotec, MCA275, 1:2000) was used to visualize liver macrophages and activated macrophages and microglia in the spinal cord. Spinal cord sections collected 0.5 cm rostral and caudal to the lesion epicenter were stained with Neurofilament antibody (DSHB, RT97, 1:2000) and Eriochrome Cyanine to visualize and differentiate axonal structure and white matter, respectively. Oil Red O staining was used to assess the deposition and accumulation of lipid molecules in hepatic tissue. Masson's Trichrome staining was used to visualize changes in liver cytoarchitecture. Slides for all stains were dehydrated using ethanol and organic solvent (Histoclear) and covered with glass held by Permount (Thermo Scientific).

Tissue Analysis and Quantification

Quantification of all histological protocols was performed by a blinded investigator with MCID imaging software (Imaging Research Inc.). Optimal sections of liver tissue were randomly selected from each group. The proportional area of CD11b+ immunoreactivity was quantified from 4-6 random fields per section and dividing the area of anti-CD11b stain by the total tissue area. Oil Red O was also quantified in this way dividing the area of Oil Red O+ stain by hepatocyte area. The injury epicenter was identified with EC/NF stained spinal cord sections and analysis of spared tissue and OX42+ macrophages/microglia were quantified at the lesion epicenter and in 9 sections spaced 200 μm rostral and caudal to the epicenter.

Analysis of Gene Expression

Frozen liver tissue was homogenized in Trizol Reagent (15596018, Life Technologies) to preserve nucleic acids. Samples were then frozen at -80 °C until assay analysis. RNA was isolated by a standard extraction protocol using chloroform/phenol[5,33]. The purity of all samples was assessed prior to amplification using microdrop spectrophotometry. The extracted RNA was then synthesized into cDNA using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Expression of specific genes adherent to studies in forthcoming sections were assessed through the use of the quantitative Real Time Polymerase Chain Reaction (Applied Biosystems). Each qPCR reaction used 100 ng of cDNA with QuantiTec primers for inflammatory genes (TNFα, CD68, CD11b, IL-1α, IL-1β) and SYBR green for detection. The amplification of each sample was normalized using Quantum RNA18S (Applied Biosystems) gene expression as a control standard. Analysis of the relative mRNA expression in each sample was determined by using the ΔΔCT method[34].

Statistical Analysis of Data

Analysis was performed by statistical software (GraphPad Prism, San Diego, CA) using ANOVA tests and post-hoc analysis to determine statistical significance. Minimum level of significance was set at a p-value less than 0.05. The Q-test for statistical outliers was utilized to confirm outliers in the data.

Results

Bile duct ligation acutely increases pro-inflammatory cytokine gene expression in the liver

We first set out to examine the effects of BDL surgery on the expression of pro-inflammatory genes, namely TNFα, IL-1β, CD11b, and CD68, at acute time points. TNFα and IL-1β are typical hallmarks in liver metabolic diseases and pathology after spinal cord injury[5,7,20,32,35,36]. Ligation of the common bile duct created a trending increase in all four cytokine genes examined at 1, 3, and 5 days post-BDL (Fig. 1). The levels of TNFα, IL-1β,

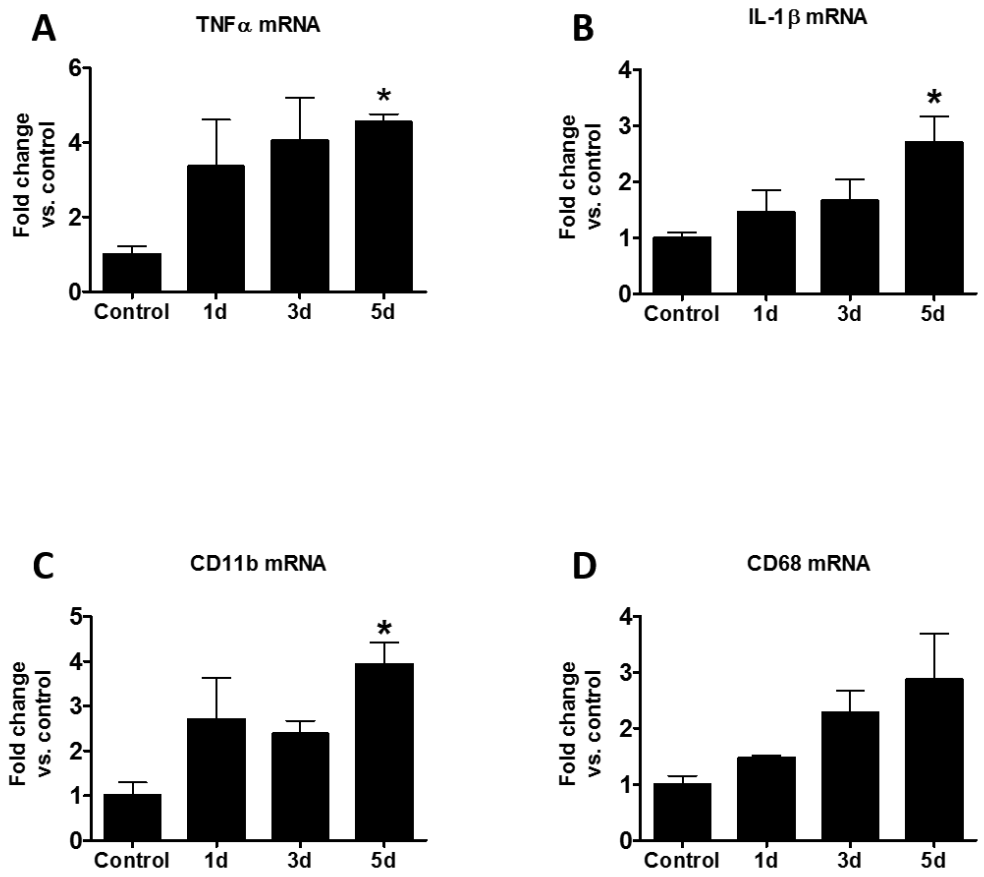

Figure 1: Elevated cytokine expression after acute ligation of the common bile duct. (A) Liver tumor necrosis factor (TNFα), (B) interleukin (IL)-1β, and (C) CD11b mRNA rose at 1 and 3 days post-injury (dpi) and were significantly increased at 5 dpi compared to sham-operated controls. (D) Liver CD68 mRNA increased at 1 and 3 dpi and was increased ~3-fold at 5 dpi, but none of these changes were statistically significant (n=2). ANOVA $p < 0.05$. *$p < 0.05$ vs. Control, n=3/group.

and CD11b were maximally and significantly increased at 5 days post-ligation compared to sham-operated controls (Fig. 1A-C). CD68 mRNA levels were increased ~2.5-fold compared to sham-operated controls at 5 days post-ligation, but this change was not statistically significant (Fig. 1D). This molecular data is evidence of the efficiency of this model to induce increased pro-inflammatory gene expression in the liver. The 5d time point presents an optimal moment to induce spinal cord injury and to examine chronic pathological changes after both BDL and SCI such as cytokine generation. These factors may then circulate in the bloodstream where they stimulate leukocyte matriculation to both the liver itself and the injured CNS[6,10,11].

Pro-inflammatory gene expression is upregulated after chronic cholestasis and SCI

Previous work from our lab showed that enhanced inflammation in the liver is evident after spinal cord injury. Cytokines TNFα, IL-1α, and IL-1β were all elevated following thoracic spinal cord injury as early as 1 dpi and as far out as 21 dpi[5]. Therefore,

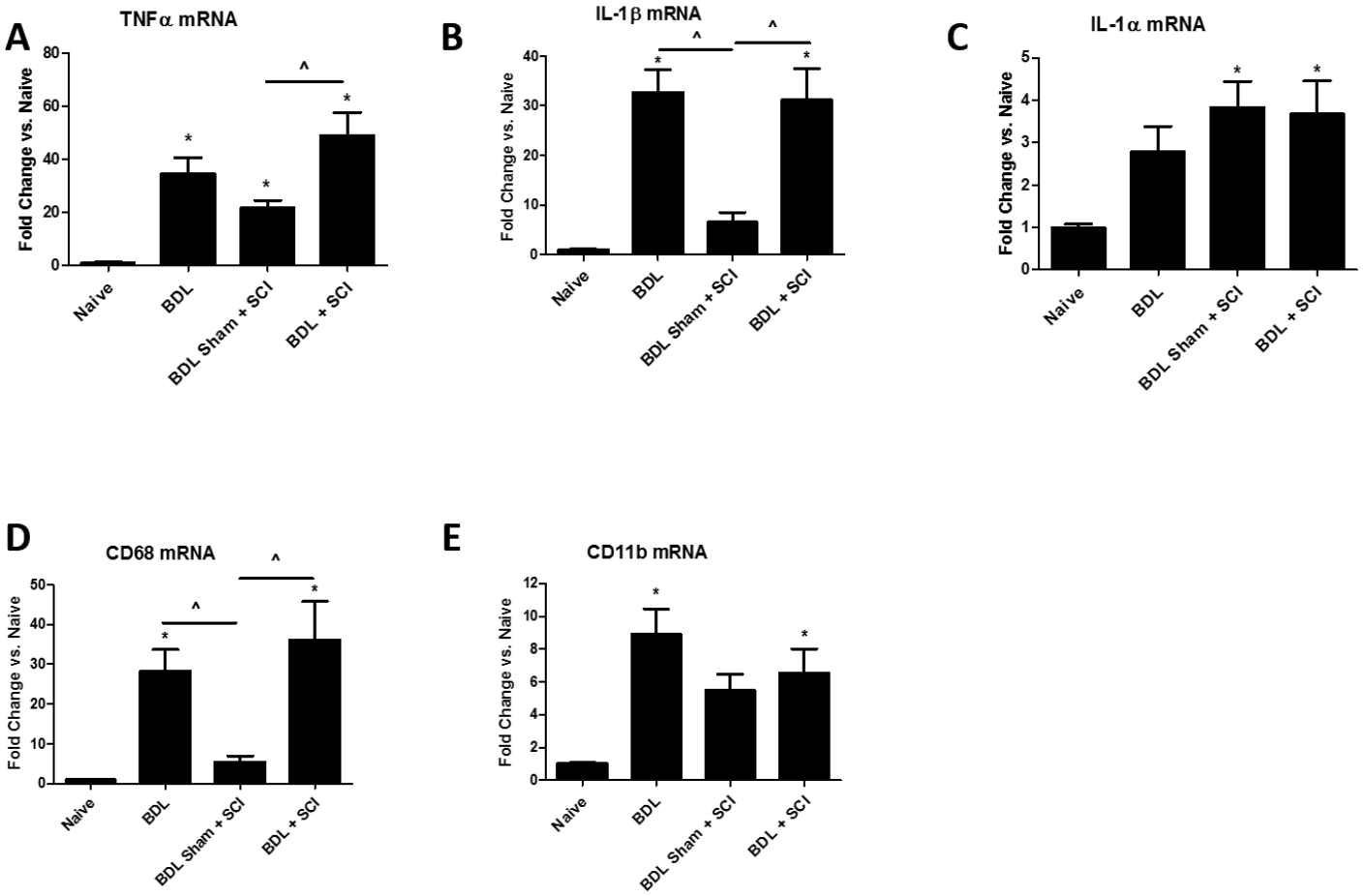

Figure 2: BDL and SCI induces chronic increases in liver pro-inflammatory gene expression. (A) Liver TNFα (B) IL-1β, IL-1α, CD68 and CD11b mRNA levels are elevated after BDL alone, BDL Sham + SCI and BDL + SCI at 23 days post SCI and 28 days post-BDL. BDL alone and BDL + SCI induced the highest increases in gene expression. ANOVA $p < 0.05$. *$p < 0.05$ vs. Naïve, ^$p < 0.05$, n=5/group.

we next compared the effects on chronic pro-inflammatory gene expression between animals that received either BDL alone, BDL Sham + SCI, or BDL + SCI. Considering that the expression of TNFα, IL-1β, and CD11b were all significantly increased at 5 days post-ligation compared to sham-operated controls, we performed BDL surgery and a T8 contusion SCI 5 days following BDL. After 28 days post-ligation, mRNA expression of TNFα, IL-1β, CD68, and CD11b was significantly increased in animals that only received BDL surgery versus naïve animals (Fig. 2A, B, D, E). Expression of TNFα and IL-1α were also significantly increased in animals that received the BDL Sham surgery + SCI at 23 days post-SCI compared to naïve controls (Fig. 2A, C). This supports the results from previous studies in our lab that showed SCI alone causes chronic inflammation in the liver[5]. The expression of all pro-inflammatory genes examined was significantly increased in the BDL + SCI group at 23 days post-SCI and 28 days post-BDL compared to naïve animals (Fig. 2A-E). This indicates the two injury models compounded chronic expression of these genes in the liver. mRNA expression of TNFα, IL-1β, and CD68 (Fig 2A, B, D) was also elevated

chronically compared to the changes seen after BDL Sham + SCI. Inducing an enhanced hepatic inflammatory state after BDL and SCI—specifically due to the pro-inflammatory Kupffer cells, cytokines synthesized within the hepatic tissue, and enhanced leukocyte entry into the liver parenchyma—confirms liver injury occurring after SCI alone is enhanced in this paradigm.

Bile duct ligation and spinal cord injury increase the quantity of hepatic macrophages

Previous data from our lab demonstrated that macrophages are increased in the liver within 2 h of experimental SCI and remain elevated out to 21 days post-SCI[5].

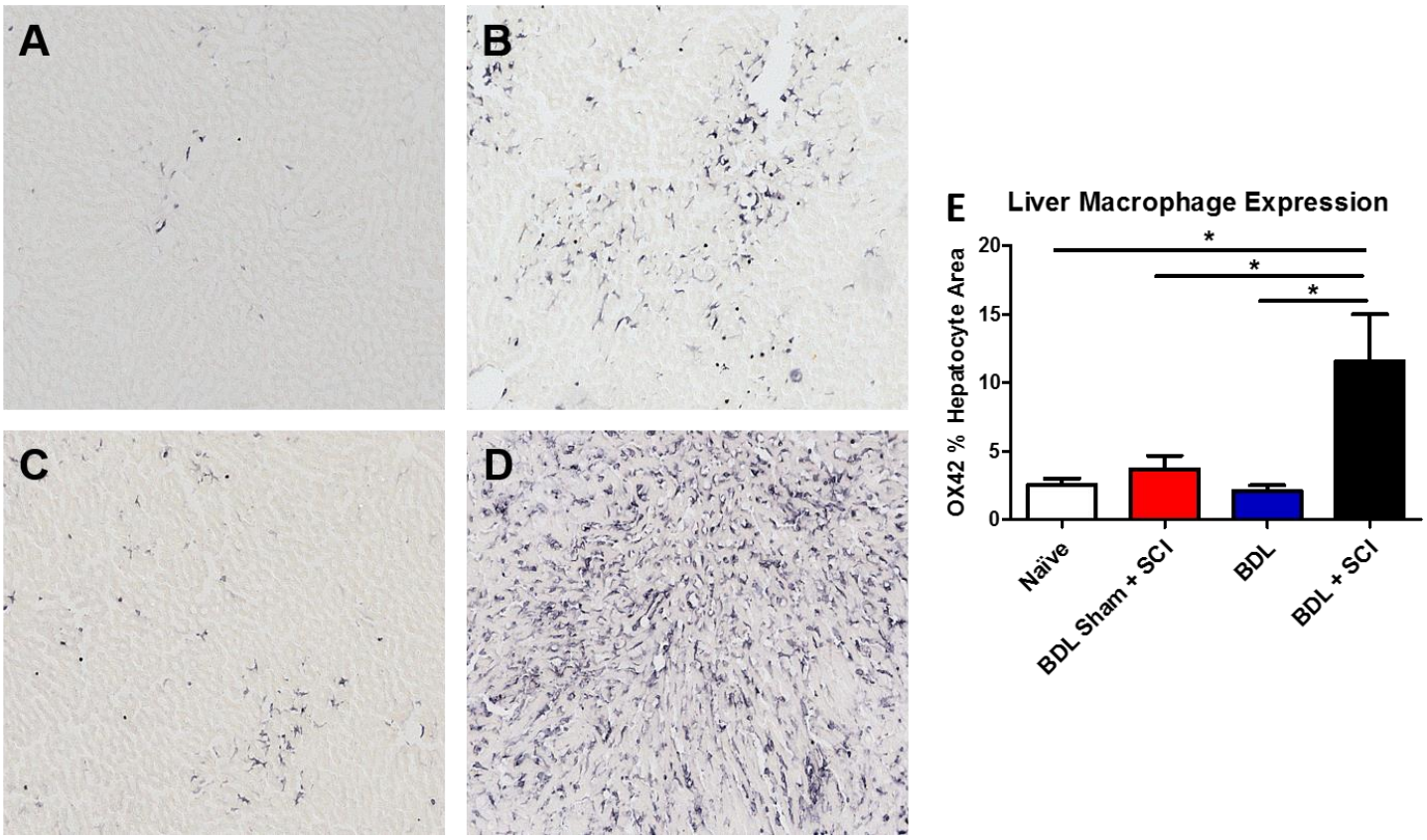

Figure 3: Hepatic OX42 (CD11b) immunoreactivity increased after induced chronic cholestasis and SCI. Representative images of OX42+ staining in the liver parenchyma from (A) Naïve, (B) BDL Sham + SCI, (C) BDL and (D) BDL + SCI at 23 days post-SCI and 28 days post-BDL. (E) Quantification of the hepatocyte area coverage of OX42+ staining. Immunoreactivity increased significantly after BDL + SCI and was unchanged compared to Naïve livers in the BDL and BDL + SCI animals. ANOVA $p<0.05$. *$p<0.05$, n=5-7/group.

Therefore, we determined if BDL + SCI animals presented with increased Kupffer cells in the liver. Kupffer cells were visualized via immunolabeling with OX42 (CD11b). CD11b expression was significantly increased ~10-fold versus naïve controls in animals that received BDL + SCI (Fig. 3D, E). Surprisingly, no significant changes in CD11b were seen in

either the BDL or BDL Sham + SCI groups compared to naïve controls (Fig. 3B, C, E). This is an interesting result that differs from previous findings that show use of CD68 immunolabeling in the liver increases ~100 fold by 21 days post-SCI[5].

Liver fibrosis is enhanced following BDL and SCI

In NASH pathology, accumulation of lipids by hepatic tissue leads to the propagation of stellate cells and irreversible fibrosis in the liver[37]. Bile duct ligation-treated rats exhibit cholestasis, increased systemic oxidative stress, and liver fibrosis, which ultimately lead to liver cirrhosis. Fibrosis is due to the proliferation of collagen-expressing hepatic stellate cells, typically developing 4-6 weeks after BDL in Sprague Dawley rats[38]. The current study showed a vast upregulation of fibrotic scarring in the liver in the BDL + SCI cohort compared to the other experimental groups (Fig. 4A-D). The BDL group only showed slight signs of increased liver fibrosis (Fig. 4C). This suggests that performing SCI after inducing hepatic inflammation exacerbates and possibly expedites collagen formation and fibrosis.

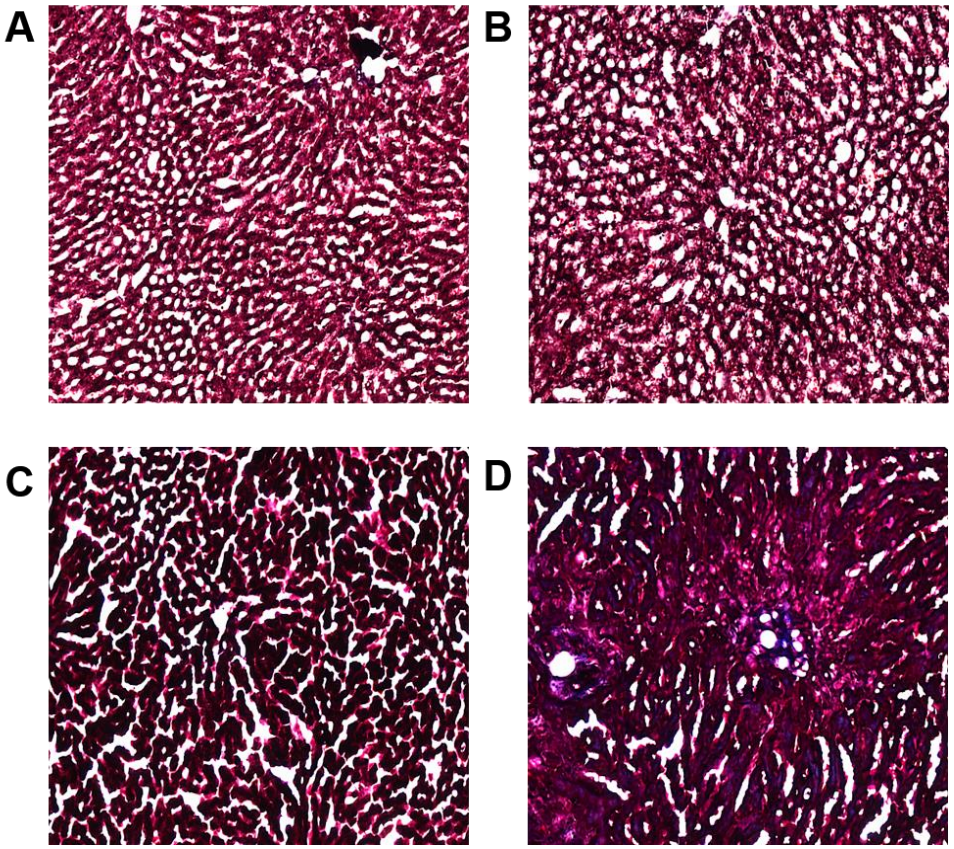

Figure 4: Extensive fibrosis in BDL + SCI livers. Representative images of Masson's Trichrome staining in the liver from (A) Naïve, (B) BDL Sham + SCI, (C) BDL, and (D) BDL + SCI at 23 days post-SCI and 28 days post-BDL. (B) BDL Sham + SCI did not induce changes in liver cytoarchitecture or collagen deposition compared to Naïve livers. Beginning stages of collagen production and fibrosis were observed in (C) BDL livers. Advanced fibrosis and alteration of the liver cytoarchitecture were observed in (D) BDL + SCI livers.

Chronic cholestatic inflammation changes expression of intrahepatic lipids following SCI

Previous studies from our lab characterized that lipids were deposited as early as 1d

and maintained through 21 days post-injury after thoracic SCI[5]. The presentation of fatty liver tissue is inherent to metabolic syndrome and NAFLD/NASH in SCI, and enhanced lipid accumulation in hepatic tissue was maintained chronically in these studies after all types of

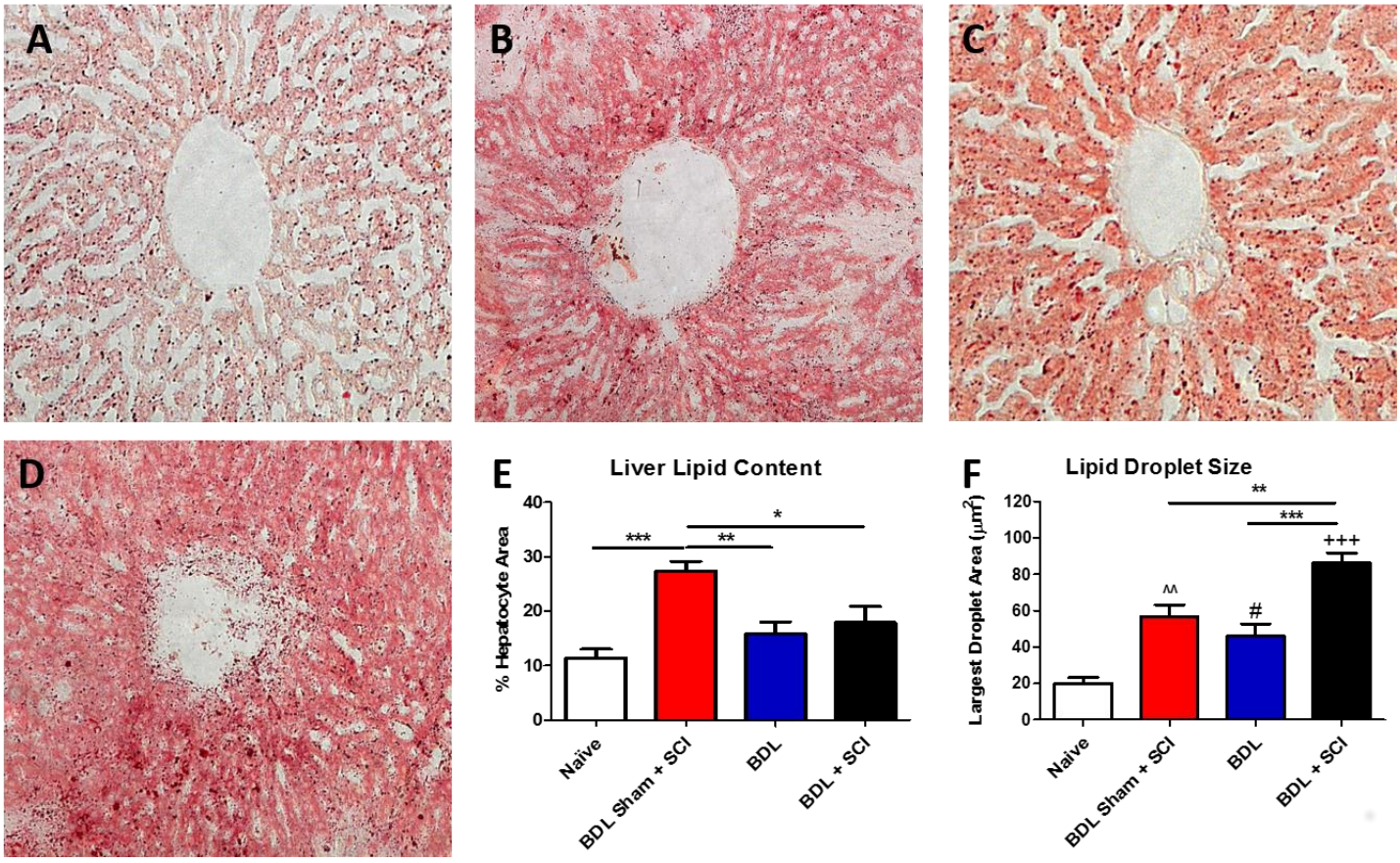

Figure 5: Increased lipid accumulation in the liver after BDL + SCI. Representative images of Oil Red O staining around vasculature in livers from (A) Naïve, (B) BDL Sham + SCI, (C) BDL, and (D) BDL + SCI at 23 days post-SCI and 28 days post-BDL. (E) Quantification of the hepatocyte area coverage of positive lipid staining. Oil Red O staining increased significantly after BDL Sham + SCI and was unchanged from Naïve livers in the BDL and BDL + SCI animals. (F) Quantification of Oil Red O+ droplet size. Droplet size was significantly increased in each group compared to Naïve and in BDL + SCI livers compared to BDL Sham + SCI and BDL livers. ANOVA $p<0.05$. ^^$p < 0.01$, #$p<0.05$, and +++$p<0.001$ vs. Naïve. *$p<0.05$, **$p<0.01$, ***$p<0.001$, n=5-7/group.

injury. The presentation of lipid retention or steatosis in the intrahepatic parenchyma is a hallmark of NASH pathology, and Kupffer cells aid in directing the inflammatory response causing hepatocyte damage and fibrosis. This is relevant to SCI patients as this pathology is evident due to their excessive weight gains and elevated levels of hepatic proteins[39]. Lipid droplet size, visualized by Oil Red O labeling, was significantly increased in all experimental groups over naïve controls (Fig. 5B, C, D, F), with the largest increase seen in the BDL + SCI group, which was ~20% larger than the BDL and BDL Sham + SCI groups. Despite the

increase in lipid droplet size in the BDL + SCI group, the overall Oil Red O distribution, measured by percent of hepatocyte area centralized around liver vasculature, was only significantly increased in the BDL Sham + SCI group (Fig 5B, C, D, E). These data support previous findings from our lab that showed SCI alone caused chronic increases in liver steatosis[5]. Despite the lack of an overall change in lipid deposition, the enhanced lipid droplet size seen following BDL and SCI may contribute to the liver's role in the acute phase of SCI leading to overreactivity of Kupffer cells during the hepatic acute phase response and pronounced intraspinal inflammation.

Induced hepatic inflammation does not exacerbate intraspinal inflammation or lesion size

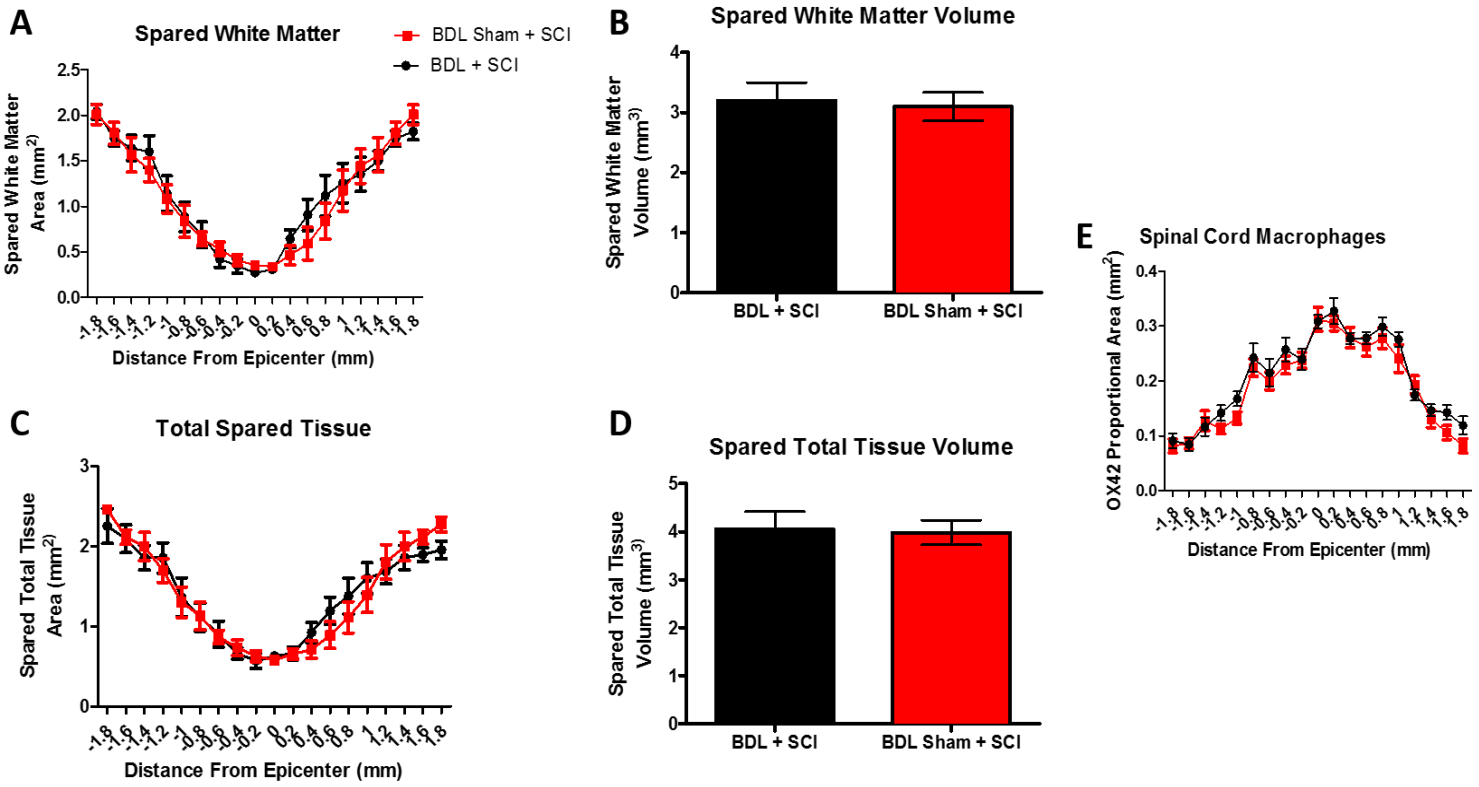

Figure 6: Cholestasis of the common bile duct did not alter intraspinal tissue pathology after SCI. (A, B) Spared white matter and (C,D) total tissue area and volumes measured via EC/NF staining were not significantly different between BDL Sham + SCI and BDL + SCI spinal cords at 23 days post-SCI and 28 days post-BDL. (E) OX42+ immunolabeling was not significantly different between BDL Sham + SCI and BDL + SCI spinal cords; n=7/group.

Liver-induced inflammation triggers disturbances in the CNS as well as metabolic and behavioral dysfunction[12]. We hypothesized that increased liver inflammation via BDL before a T8 contusion SCI would exacerbate the histological deficits seen in the spinal cord 28 days post-ligation and 23 days post-SCI. Surprisingly, Eriochrome Cyanine staining revealed no significant differences in spared white matter area and volume (Fig. 6A, B) or

total tissue area and volume (Fig. 6C, D) between the BDL Sham + SCI and BDL + SCI groups. Thus, Kupffer cell "gain of function" via induced hepatic inflammation did not exacerbate the lesion size of the injured spinal cord as was expected. Extravasation of hematogenous macrophages and activated resident microglia within the lesion are established characteristics of spinal cord injury[7,40]. Immunohistochemical staining revealed no significant differences in OX42+ macrophages/microglia between BDL Sham + SCI and BDL + SCI groups (Fig. 6E). Contrary to our predictions, no changes in secondary pathological events following SCI occurred when induced hepatic inflammation preceded spinal cord trauma.

Gadolinium chloride selectively reduces Kupffer cells in the liver

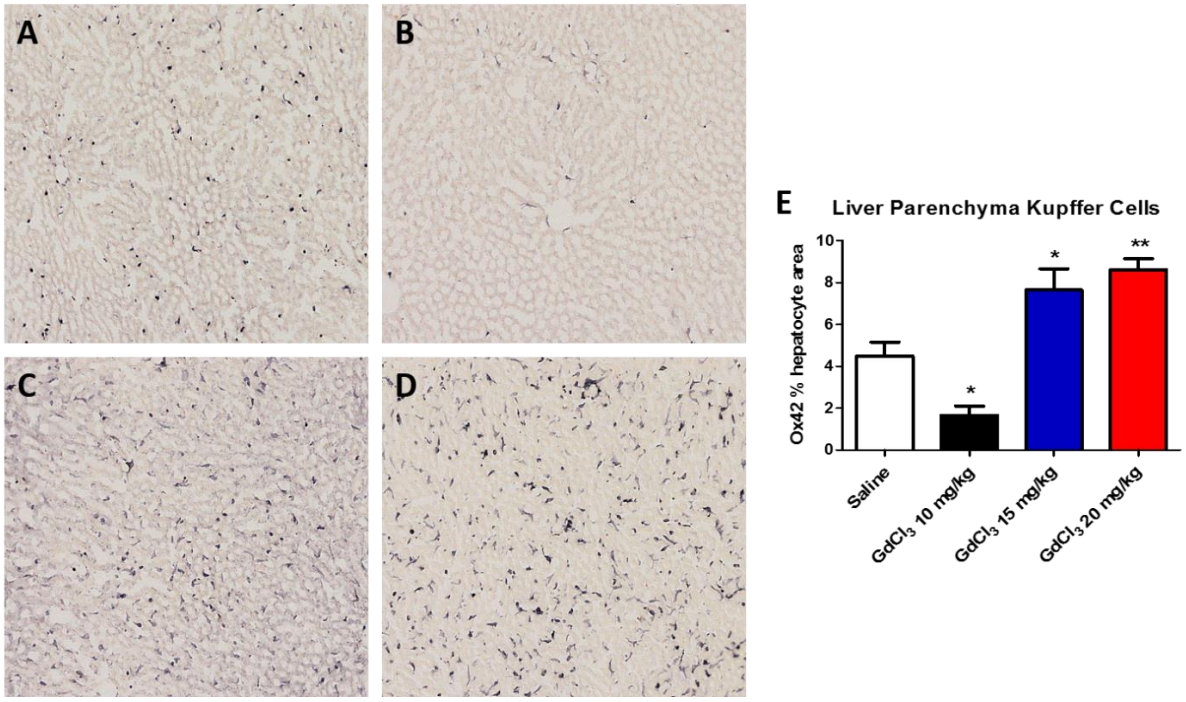

Figure 7: Intravenous $GdCl_3$ reduces Kupffer cells. Representative images of OX42 staining in the liver parenchyma from (A) Saline, (B) 10 mg/kg $GdCl_3$, (C) 15 mg/kg $GdCl_3$ and (D) 20 mg/kg $GdCl_3$ 48 hours after injection. (E) Quantifiction of OX42+ staining in the liver parenchyma. The 10 mg/kg $GdCl_3$ dose significantly reduced OX42 immunoreactivity in the liver parenchyma by ~65% vs saline-injected controls. 15 mg/kg and 20 mg/kg $GdCl_3$ doses increased OX42+ immunoreactivity by ~35% and ~40%, respectively, compared to saline-injected controls. ANOVA $p<0.05$. *$p<0.05$, **$p<0.01$ vs saline, n=6 for Saline and $GdCl_3$ 10 mg/kg; n=3 for $GdCl_3$ 15 and 20 mg/kg.

The hepatic APR contributes to the inflammation following injury to the CNS, and attenuating Kupffer cell activation may reduce this phenomenon[8,14]. The compound gadolinium chloride ($GdCl_3$) eliminates liver Kupffer cells when introduced into the bloodstream. Despite the negative results of the BDL + SCI "gain of function" experiment in

the post-traumatic spinal cord, we hypothesize that decreasing liver inflammation before SCI will attenuate outcomes after SCI. Pilot studies were conducted to determine an optimal dose of $GdCl_3$ that effectively reduces Kupffer cell numbers 48 hours after lateral tail vein adminstration. 10mg/kg of $GdCl_3$ reduced OX42+ cells in the liver parenchyma by ~60% compared to saline-injected controls (Fig. 7B, E). The 15 and 20 mg/kg doses surprisingly led to ~35% and ~65% increases in Kupffer cell numbers 48 hours after injection, indicating that these higher doses caused a robust depletion and possibly quicker, compensatory repopulation of Kupffer cells (Fig. 7C, D, E). None of the doses examined affected the numbers of OX42+ cells in the spinal cord (data not shown). This result is concordant with multiple other studies attenuating insult to the liver administering this efficacious dose[14,24,30,41]. Thus, Kupffer cell "loss of function" is achieved with $GdCl_3$ administration and future studies will determine if this depletion has an effect on the acute outcomes of SCI pathology.

Discussion

The current studies were designed to determine how alterations in hepatic inflammation affected the pathogenesis of spinal cord injury and subsequent peripheral sequelae. Multiple studies demonstrate hepatic inflammation occurs prior to and facilitates the dramatic inflammatory response after CNS injury[6,8,10,13,14,16]. Therefore, we investigated whether altering the hepatic acute phase response—in "gain of function" or "loss of function" experiments—produced any effects post-SCI. We showed that ligation of the liver common biliary duct induces the production of pro-inflammatory cytokines that reach peak levels in the liver 5 days post-ligation. A T8 contusive spinal cord injury during this peak time of liver inflammation lead to increases in chronic expression of hepatic pro-inflammatory cytokines, Kupffer cell numbers, fat deposition, and fibrosis compared to animals that either underwent BDL or SCI alone. These pathological changes are concomitant with nonalcoholic fatty liver disease or NAFLD prevalent in human SCI and as a precedent to nonalcoholic steatohepatitis or NASH. Despite the apparent compounding effect of the contused spinal cord in the already damaged liver, chronic cholestasis surprisingly did not lead to significant changes in spinal cord pathology compared to animals subjected to SCI alone. In addition, an optimal dose of $GdCl_3$ was established to selectively decrease Kupffer cell numbers in the liver parenchyma. Future studies will utilize this compound with SCI to test whether downregulating the hepatic APR could improve post-SCI repercussions. Understanding the inflammatory mechanisms of hepatic inflammation in CNS injury and vice versa could lead to therapeutic interventions in the future that improve clinical SCI outcomes.

Preliminary experiments displayed that bile duct ligation increased hepatic pro-inflammatory gene expression at 5 dpi. mRNA levels were increased for TNFα and IL-1β,

leading credence to BDL as an effective model that induces a prolonged, sustained inflammatory hepatic environment[28,42,43]. The hepatic APR stimulates the production of cytokines following trauma from multiple etiologies, and it is established that TNFα and IL-1β are synthesized prior to later phase mediators such as IL-6 or transforming growth factor β (TGFβ)[9]. TNFα especially is important in triggering the production of other mediators promoting inflammatory cell recruitment, hepatocyte death, and fibrogenesis[44]. These soluble factors are synthesized by KCs as a response to inflammatory diseases, increasing leukocyte number in the liver parenchyma as an initial reaction after SCI or any other traumatic event[5,11,16]. This phenomenon was supported in our experiments as CD11b mRNA significantly increased 5 days post-BDL. Marked increases in macrophage mRNA after BDL indicates an acute inflammatory response following hepatocellular insult. Thus, pro-inflammatory gene expression was effectively elevated using BDL at 5 dpi, and it was concluded this was an ideal time point to use in our studies of cholestasis-induced hepatic inflammation and thoracic spinal contusion.

Chronic cholestasis and SCI produces robust changes in hepatic cytokine mRNA synthesis, indicated by the significant increase in their transcription versus other experimental groups. Previous studies show that after thoracic SCI alone, hepatic inflammatory cytokine mRNA of TNFα was significantly increased by 21 dpi[5]. Our results demonstrated significant increases in TNFα, IL-1β, and CD68 transcripts 23 days after SCI in the BDL + SCI livers. These findings support previous work arguing prolonged hepatic cytokine expression after SCI contributes to dysfunctional liver and NAFLD/NASH pathologies at chronic time points in models of cervical, thoracic, and lumbar SCI[5]. Cholestasis alone caused increased mRNA levels of all pro-inflammatory cytokines examined at 28 days post-BDL compared to naïve. The BDL + SCI cohort yielded the highest expression of cytokine mRNA versus naïve and Sham + SCI animals. This indicates that the upregulation of inflammatory gene transcription due to chronic cholestasis is exacerbated by spinal contusion, adding to the growing body of evidence arguing cross-talk exists between the hepatic and CNS inflammatory responses[6,8,12–14]. In the BDL + SCI double injury model, the "cytokine storm" produced by liver Kupffer cells, stellate cells, and hepatocytes assists in the enhancement of leukocyte entry guiding intrahepatic inflammation and tissue injury. Elevated levels of these soluble factors may also circulate in the bloodstream and recruit leukocytes to the injured spinal cord[6,11,32]. Indeed, the inflammatory environment of the liver is increased in "gain of function" experiments indicating effects of this prolonged inflammation are detrimental to the health and lifespan of the SCI population.

Liver histology experiments revealed in the BDL Sham + SCI group that hepatic OX42 (CD11b) immunolabeling is increased at 23 days post-SCI, again supporting the idea that SCI contributes to chronic hepatic inflammatory changes. Increased OX42+ macrophages in livers from animals that underwent BDL before SCI indicates an increase in Kupffer cells and hematogenous macrophages infiltrating the liver parenchyma. The

differences observed by our group in cytokine expression and influx of leukocytes to the liver after CNS trauma are similar to work from other groups[6,13,45] and reinforces the argument that SCI can influence hepatic inflammatory respones. The pathology of accumulating macrophages and prolonged hepatic cyokine mRNA expression further indicates enhanced inflammation post-SCI predisposes NAFLD/NASH-like symptoms from dysregulation of liver metabolism in a model of chronic liver inflammation and spinal contusion. The activation of pro-inflammatory Kupffer cells in NAFLD/NASH is a topic that is constantly being investigated. In the fatty liver KCs are viewed as a primary source of cytokine and chemokine production because steatotic hepatocytes in NAFLD release modified lipoproteins that can activate KCs inducing an inflammatory response[37]. Our group previously found one particular group—ceramides—increased within the liver after SCI. These lipids are important signaling molecules thought to activate Toll-like receptor-4 (TLR4) located on KCs and hepatocyes perpetuating NASH-like pathology[5]. It has also been postulated that apoptotic hepatocytes contribute to this activation by inducing inflammasome complexes in liver macrophages[46]. Inflammation in the NASH liver amplifies from various sources of lipids including accumulating free fatty acids (FFAs) in the circulation or endotoxins such as LPS and facilitates hepatocyte apoptosis, stimulation of mononuclear immune cells, and concordantly cytokine synthesis[46–48].

Hepatic fibrosis was increased and persisted chronically when experimentally induced hepatic inflammation preceded thoracic SCI. Fibrosis occurs due to activation of hepatic stellate cells within in the space of Dissé and closely associates with the hepatic epithelium and sinusoidal endothelial cells[49]. Hepatocellular injury induces stellate cells to switch from a state of quiescence to a profibrotc phenotpye. This is in part mediated by KC production of cytokine TGFβ and lipid peroxides. These products activate stellate cells and upregulates production of collagen, altering the cytoarchitecture of the liver[32,49,50]. The presentation of excessive fibrosis observed in our studies potentially stems from the excessive cytokine synthesis, lipid deposition, and macrophage infiltration seen in the hepatic parenchyma and is indicative of NAFLD/NASH-like symptomatology. In parallel with our KC data, this phenomenon is not uncommon as infiltration of KCs is shown to precede activation of stellate cells[50]. Therefore, spinal cord injury conceivably enhances hepatic innate immune cascades leading to advanced liver pathology similar to NAFLD/NASH. A plausible cause of this observation is dysfunctional fat metabolism and hepatocyte overload leading to macrophage accumulation, activation, and the resulting stellate cell-mediated fibrosis[46].

Under normal circumstances, the liver functions as a primary metabolic organ by producing bile. This substance is then transferred through the biliary tree and either drains directly into the intestinal lumen via the common bile duct or is stored in the gallbladder. Major functions of bile include the clearance of harmful endogenous or exogenous substances, absorption of dietary fats, and providing antibodies to fight enteric infections[51]. Due to factors such as obesity, insulin resistance, dyslipidemia, and chronic

inflammation, there is strong evidence that SCI patients are more likely pre-disposed to the development of metabolic syndrome and NASH in the liver[1,5,52]. Steatosis in the liver is defined as disordered lipid metabolism and accumulation triggering oxidative stress, inflammation, and hepatocellular damage[53,54]. Following thoracic SCI, accumulation of lipids in hepatic tissue can be observed as early as 1 dpi and remain elevated chronically[5]. Our data show that the BDL Sham + SCI exhibited the highest overall amount of deposition, which supports this idea that SCI can affect hepatic lipid metabolism chronically. Unexpectedly however, our experiments show that lipid accumulation following cholestasis alone and cholestasis followed by SCI is unchanged compared to naïve controls.

These results are surprising based on previous studies displaying a direct correlation between increased hepatic inflammation and lipid deposition[5]. This phenomenon may be due to the beginning signs of fibrosis seen in the BDL group and extensive fibrosis post-BDL + SCI. Nevertheless, our results still indicate disruption of metabolic homeostasis in the liver influences the vast accumulation of lipids observed in NAFLD/NASH occurring after chronic SCI[5,46,53]. Despite the BDL + SCI cohort showing unchanged overall lipid deposition compared to naïve controls, significantly larger lipid droplet sizes were observed when juxtaposed to BDL and Sham + SCI cohorts. A characteristic of steatosis in the liver is the presence of lipid droplets. Initially viewed only as inert fat storage, lipid droplets are increasingly being recognized as functional cellular organelles consisting of regulatory proteins utilized for cell signaling, lipid metabolism, and inflammation[54–56]. These cellular constituents are possibly concomitant with regulating transcriptional control of inflammation, supplementing KC activation and macrophage recruitment. It is speculated certain components within lipid droplets have the capability to activate KCs in fatty liver diseases; however, this has never been directly tested[54].

Injury to spinal cord inhibits the direct line of CNS communication to the periphery originating from pre-ganglionic sympathetic neurons in the intermediolateral cell column or descending parasympathetic pathways[22] and leads to improper communication and dysfunction. Autonomic regulation of the liver is dysfunctional after SCI, and the liver undergoes severe changes including upregulation of leukocytes and chronic pro-inflammatory cytokine synthesis, steatosis, and fibrosis as a result. Though the endogenous neuroinflammatory response after SCI is substantial, multiple reports indicate a hepatic inflammatory component precedes intraspinal inflammation and may promote recruitment of peripheral monocytes to the site of injury[6,8,11,16,20]. It was postulated that upregulating liver inflammation prior to spinal contusion would elevate inflammation after SCI and in turn exacerbate damage in the spinal cord. However, there were no discernable differences in spared total tissue or spared white matter tissue between the Sham + SCI and BDL + SCI cohorts. We also demonstrated that hepatic APR “gain of function” experiments did not change expression of macrophages/microglia in the spinal cord lesion epicenter. The influx of hematogenous leukocytes and microglial activation contribute to destruction

of the blood-spinal cord barrier and secondary tissue damage observed after traumatic SCI[7,18,57]. Removal of hematogenous macrophages from the lesion epicenter showed promise in previous work as a therapeutic intervention[58]. Other studies using bone marrow chimeric mice showed peripheral leukocyte entry into the contused spinal cord occurs[40] maximally at 7 dpi[18]. The current findings suggest no significant pathological changes in the lesion occur if hepatic inflammation precedes spinal cord injury.

The BDL model itself potentially is the culprit behind the unexpected lack of altered spinal cord pathology in the BDL + SCI group. It is important to remember the hepatic APR contributes to and possibly precedes intraspinal pathology, but there is still an endogenous SCI inflammatory cascade predominating at the site of the lesion[6,57]. The BDL surgery is effective at increasing inflammation in the liver, yet the downside is the severe pathological changes that it induces in the liver, such as advanced fibrosis, which could be impacting liver function at an extreme level. Future studies will be needed to elucidate an alternative model that targets inflammatory cells while also limiting the amount of stellate cell activation and subsequent liver fibrosis. Two options include using either a shorter BDL time point than 28 days or to employ a reversible BDL model[59]. Fibrosis typically forms in livers anywhere from 14 to 28 days following BDL. Utilizing a shorter time point or a reversible BDL model could eliminate any confounds the advanced fat deposition and fibrosis create while still taking advantage of the inflamed liver environment. One group also reported an effective murine model for acute hepatitis B that subsides after 7 days post-injection via tail vein[60]. Transfection with this type of acute virus could provide a suitable inflammatory response in the liver that may enhance the APR after SCI. If alternative methods do not elicit exacerbated SCI pathology, it could reveal that the hepatic APR reaches a threshold contribution following CNS injury. Essentially this means the liver differentially contributes to the secondary damage occurring after SCI when compared to the endogenous spinal cord response. Even so, this does not indicate the hepatic APR is devoid of a role in the cascade of neuroinflammatory events after SCI.

The Kupffer cell reducing compound $GdCl_3$ decreased hepatic parenchymal macrophages 48 hours after IV injection at an optimal dose of 10 mg/kg. This preliminary finding indicates significant KC knockdown in the liver is possible, yet further analysis of hepatic cytokine gene expression and histological consequences are required. Future experiments will couple SCI at 48 hours after $GdCl_3$ injection to analyze the effect of KC depletion on the lesion in the spinal cord and resulting pathology in the liver. Recent studies reveal that Kupffer cell knockdown prior to SCI reduced neutrophils recruited to the contused spinal cord by ~50% using clodronate liposomes[8]. Reducing KC numbers using this same technique also proved to reverse hepatic steatosis and macrophage recruitment in the NASH liver[47]. Therefore, it can be said that targeting this specific cell population may potentially reduce the inflammatory response in the injured CNS and reduce secondary damage as a result of this attenuation. KC knockdown may also quell

fibrosis in the liver as these cells are important synthesizers of pro-fibrotic factors like TGFβ and facilitate activation of hepatic stellate cells[32]. By inhibiting KC responses, the vicious cycle perpetuating liver damage and inflammation after spinal cord injury could be inhibited and lead to therapeutic improvements.

Altered peripheral outcomes of traumatic SCI ensue after chronic cholestasis and thoracic spinal contusion in rat models. The array of changes in pro-inflammatory cytokine gene expression, macrophage expression, lipid accumulation, and fibrosis in the liver contribute further evidence to the concept of hepatic inflammation preceding and exacerbating peripheral damage after intraspinal trauma. These findings supplement previous work revealing SCI leads to symptoms concomitant with nonalcoholic steatohepatitis, a chronic health condition due to metabolic syndrome in clinical cases. Although spinal cord pathology was not altered by cholestasis-induced hepatic inflammation, further analyses will need to be carried out to determine if augmenting hepatic inflammation prior to SCI influences post-traumatic after effects. Attenuation of macrophage levels in the liver parenchyma reveals the exciting potential to improve outcomes following experimental SCI. A physiologically important metabolic and immunological organ, the liver is a fascinating therapeutic target to study. Diminishing its role as both victim and malefactor of CNS injury may improve quality of life, health, and well-being in future clinical cases of human SCI.

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This work would not have been possible without the support of my entire family during my undergraduate education, and I thank them for always believing in me.

I dedicate this work to my family and my late grandfather Wade Gilliland.